Biology and Fertility of Soils
Volume 55, 649-659 (2019)
https://doi.org/10.1007/s00374-019-01384-5

Allelopathic effects account for the inhibitory effect of field-pea (*Pisum sativum* L.) shoots on wheat growth in dense clay subsoils

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Abstract
The deep-placement of nutrient-rich organic amendments in poorly-structured subsoils can improve subsoil structure and increase grain yields, but its widespread adoption by farmers is limited by the availability and cost of animal manures, the current choice of amendment. Three glasshouse experiments investigated the effectiveness of dried field pea **(***Pisum sativum* L.) shoots (green chop), as green manure, on wheat growth in three subsoils with contrasting soil chemical and physical properties. The growth of wheat plants was greatly suppressed when the green chop was placed in Sodosol and Chromosol subsoils. In contrast, there was a 2-fold increase in shoot biomass in response to the addition of green chop to Vertosol. Three allelopathic compounds, pisatin, anhydropisatin, and maackian, were identified at higher concentrations in the extracts of remaining green chop residues in the Sodosol and Chromosol, compared to the Vertosol, directly supporting phytotoxicity as the cause of observed inhibitory effects of green chop in these soils. The persistence of the phytotoxicity in the Sodosol might be attributed to its poor aeration caused by poor structure or compaction. Nevertheless, pre-incubation led to microbial decomposition of the allelochemicals in the Sodosol, though at a much slower rate than in the Vertosol. Further studies are needed to determine the time period required for the disappearance of the phytotoxic effects in soils with different physico-chemical properties.

Key words: allelopathic effects, deep placement, green chop, LC-MS/MS, organic amendments, dense clay subsoils

Introduction
Subsoil constraints, such as high bulk density and high sodicity, reduce crop productivity worldwide. Subsoil manuring is a farming practice developed in Australia to improve the structure and aeration of dense clay subsoils. The current practice involves the deep-placement of high rates of nutrient-rich organic manures in rip-lines in the upper layers of the subsoil. Yield increases have been reported following the intervention, in the high-rainfall cropping regions of south-eastern Australia (Gill et al. 2009; 2012). The success of subsoil manuring practice has been attributed to its effectiveness in supplying nutrients, in improving soil porosity and permeability in the clay subsoil, in stimulating deep root proliferation, and in increasing subsoil water uptake (Armstrong et al. 2007; Gill et al. 2008; 2009).

Subsoil manuring is an expensive practice if the organic materials are not sourced locally. An economic analysis by Sale and Malcolm (2015) has found that the major costs in the practice are in purchasing, transporting and handling the large quantities of organic amendments. The cost of the poultry litter, one of the amendment choices, is increasing and will continue to increase along with the high demand. There is a need to identify cheaper and more realistic organic amendments to replace the animal-based amendments, in order to promote the adoption of the practice on a commercial scale. Interest is now developing in the use of plant biomass as subsoil amendments, as it can be grown and sourced locally on the farm. Potential choices for such amendments include cereal straws, or custom-grown green manure legume crops (referred to as 'green chop') which can be harvested at peak biomass stage and incorporated in the subsoil.

There is little information available on the yield benefit of such plant biomass amendments incorporated at high rates in the dense clay subsoils. The effectiveness of surface-applied plant biomass amendments on crop yield, however, has been extensively studied. For example, surface application of wheat straw reduced crop yields, possibly due to decreased N availability via net microbial N immobilization (Schoenau and Campbell 1996; Morris et al. 2010), but increased crop yields when it was applied with fertilizer nutrients (Kushwah et al. 2016). Green manures with low C:N ratio (<20) are likely to mineralize rapidly and increase N availability to the crop (Green and Blackmer 1995; Dinnes et al. 2002). Therefore, the incorporation of legume green manure offers an effective way in improving soil N fertility and in increasing crop yield (Fageria, 2007; Wortman et al. 2017). On the other hand, some of the crop residues can effectively suppress the growth of weeds (Rice 1984; Alkhatib et al. 1997; Krishnan et al. 1998) or other

crops (Patrick 1971; Rice 1984) via release of phytotoxic compounds, known as allelochemicals. Bonanomi et al. (2006) found that the shoots of 22 out of 25 plant species showed phytotoxic effects during their decomposition, and concluded that the phytotoxicity of plant materials is a general phenomenon rather than a special case. The phytotoxic effect of crop residues in soil did not persist for a long period when they were surface incorporated, due to rapid degradation of allelochemicals into nontoxic molecules by microbes (Harper 1977; Kaur et al. 2009; Ehlers, 2011). Dense clay subsoils often exhibit poor aeration when the clay is moist (MacEwan et al. 2010), which may deliver an unfavorable condition for microbial decomposition of either amendments or allelochemicals. Therefore, it is difficult to predict how effective plant biomass amendments will be in subsoils with different physical and/or chemical properties, relative to the topsoil.

This paper documents the results of three column experiments undertaken to investigate the effectiveness of wheat straw and dried field pea shoots (green chop) on wheat growth, when wheat straw and green chop were incorporated in the subsoils. Three soils used were a Chromosol, Sodosol and Vertosol with contrasting physical and chemical properties in subsoils. The aim of the second experiment was to understand the unexpected results in the first experiment where minimal root growth occurred in the green chop-amended Chromosol and Sodosol subsoils. We tested hypotheses that the suppression of root growth in the Sodosol and Chromosol subsoils resulted from nutrient limitations or from toxic compounds released from the decomposing green chop shoots. A liquid chromatography tandem-mass spectrometry (LC-MS/MS) technique was used to determine if phytotoxic compounds were present in the extracts of decomposing green chop material. A third supplementary column experiment determined whether soil pH, aeration or the duration of a pre-incubation period, affected the green chop-induced root suppression of wheat plants in the Sodosol subsoil.

Materials and Methods

Soils and organic amendments

Topsoil (0-10 cm) and subsoil samples (20-40 cm) of three soils, classified as Sodosol, Chromosol and Vertosol (Isbell, 2002) or Solonetz, Luvisol and Vertisol (IUSS Working Group (2015), were collected from farmer's fields in south-west Victoria, Australia. These soils are representative of the major cropping soil types in Victoria. The Sodosol subsoils were sodic with an exchangeable sodium percentage (ESP) of 11%, while the Vertosol and Chromosol were non-sodic (ESP < 2%). All soils were air-dried and sieved (<2 mm).

The poultry litter amendment was collected from a broiler farm in country Victoria. Wheat straw was collected in the field when wheat crop was harvested, while field peas shoots (green chop or GC) were cut off at ground level at the flowering stage and subsequently air-dried. Wheat straw and green chop were chopped or ground through a 6-mm sieve. All organic materials were dried in the oven at 70 °C for 2 days before being analyzed for total C, N, P and K (Table 2).

Experimental set-up

The experimental unit was a 45-cm high PVC column with an internal diameter of 10 cm, and sealed at the base with a PVC cap. The soil profile consisted of 10-cm of topsoil, overlying 32-cm of subsoil, which was packed layer by layer with consistent tapping and watering. The final bulk density in the subsoil was 1.4 g cm^{-3} for the Sodosol and Chromosol and 1.3 g cm^{-3} for the Vertosol. The organic amendments were evenly spread across the column, on top of 25 cm of subsoil (7 cm below the bottom of the topsoil i.e. at a depth of 17 cm from the soil surface). Basal nutrients were added to the topsoil at the following rate (μg g^{-1}): KH_2PO_4, 180; K_2SO_4, 120; $CaCl_2.2H_2O$, 180; $MgSO_4.7H_2O$, 50; $MnSO_4.H_2O$, 15; $ZnSO_4.7H_2O$, 9; $CuSO_4.5H_2O$, 6; $Na_2MoO_4.2H_2O$, 0.4; FeEDTA, 5.5. The soil surface was covered with a 3-cm layer of plastic beads to prevent surface evaporation. A central watering tube was inserted into the clay subsoil to add water at depths of 14 to 35 cm into the subsoil.

Experiment 1 involved 45 columns and was set up in a glasshouse with conditions set at 25 °C during the day and 18 °C during the night. This study involved the factorial combination of five treatments (control, green chop, wheat straw, wheat straw + fertilizer nutrients (straw+NPKS), and poultry litter) and three soils (Chromosol, Sodosol and Vertosol), with each of the 15 treatment combinations being allocated to 3 blocks in a randomized complete block design. All organic amendments were added on an equivalent surface area basis of 20 t ha^{-1} (15.7 g $column^{-1}$). Fertilizer nutrients were mixed through the chopped wheat straw in a liquid form, at the rate of 0.418 g DAP, 0.32 g urea and 0.526 g K_2SO_4 $column^{-1}$, for the straw+NPKS treatment, which were equivalent to 300 kg N, 125 kg P, and 300 kg K per ha, on a surface area basis. All soil columns were wet to 80% of field capacity and pre-incubated for one month at 25 ºC, before sowing the wheat plants.

Experiment 2 involved 36 columns and was conducted in a Controlled-Environment Room (CER) with conditions set to a 14-h day at 23 °C and a 10-h night at 18 °. The light intensity was 450 µmol m^{-2} s^{-1}. The treatments involved the factorial combination of four amendments (control, green chop, green chop+NPKS and NPKS) placed in the subsoil of the three soils (Chromosol, Sodosol and Vertosol), with each treatment replicated three times in a randomized block design. The fertilizer nutrient treatment (NPKS) received 0.418 g DAP (di-ammonium phosphate), 0.32 g urea and 0.526 g K_2SO_4 $column^{-1}$ in solution, which was equivalent to 300 kg N, 125 kg P, 300 kg K, and 123 kg S per ha on a surface-area basis. The green chop+NPKS treatment had the fertilizer nutrients added in the liquid form, at the same rates as the NPKS treatment. The green chop was applied at the rate of 15.7 g $column^{-1}$ which was equivalent to 20 t ha^{-1} on a surface area basis. No pre-incubation was applied in this experiment.

Experiment 3 was a 15-column experiment set up in the same CER as Experiment 2. Five amendment treatments were added in the subsoil of the Sodosol. The treatments were green chop only (GC), green chop mixed with activated carbon (GC+AC), green chop sandwiched within a 4-cm limed soil layer (GC+lime), green chop sandwiched within a 4-cm sand layer (GC+sand), and green chop pre-incubation for 10 weeks before sowing (GC+10w). The lime was added to increase soil pH to 6.5. Activated carbon was mixed with green chop at 4 g $column^{-1}$. The direct effect of activated carbon on the plant growth had been tested prior to experiment 3, by including a treatment with AC only and this had no effect on wheat growth.

Growing conditions

Eight pre-germinated wheat seeds (*Triticum aestivum* L. cv. Yipti) were sown in each column. After emergence, the plants were thinned to three per column. The water content in the soil columns was maintained at 80% of field capacity, by watering to weight every 2 days with deionized water. Supplemental N was added at 40 mg N kg^{-1} as urea to the Vertosol and Sodosol 20 days after sowing to avoid N deficiency at the early stage. Plants were harvested at day 42 at the stem elongation stage.

Measurements

The daily water-use by the wheat plants was measured by weighing all columns every two days, while tiller numbers and SPAD readings (chlorophyll index indicating canopy greenness) for the youngest fully expanded leaves were recorded every 7 days. At harvest, all soil columns were sectioned into three layers: the upper layer (0-14 cm), the amendment layer (14-20 cm) and the subsoil layer (20-42 cm). Roots at each layer were carefully recovered, washed and scanned for root length using an EPSON EU-35 scanner (Seiko Epson Corp, Suwa, Japan) and the WinRHIZO STD 1600+image analysis system (Regent Instruments, Quebec City, Canada). All plant samples were oven-dried at 70 °C and weighed. Shoots were ball-milled (Retch MM 400, Haan, Germany), and analyzed for N using a CHNS Analyzer (PerkinElmer EA2400, Shelton, CT, USA). Shoot P and K content was measured using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry) after being digested with concentrated nitric and perchloric acid (4:1).

A small soil sample located immediately above and below the amendments was collected, and stored at 4 °C for 16 h prior to determine the soil microbial biomass C (MBC) and the extractable organic C (EOC)

in the soil. The MBC was determined using chloroform fumigation-extraction according to Vance et al. (1987). The 0.5 M K_2SO_4 -extractable soil organic C (1:5, w/v), were determined by a TOC analyzer (GE Sievers InnoyOx Boulder, USA).

The remaining green-chop residues in soil were carefully recovered at the harvest and stored at -80°C for identifying allelochemicals using Exactive Plus LC/MS (Thermo Scientific). Briefly, all recovered green chop including the original material were freeze-dried and homogenized using a ball-mill grinder. To extract allelochemicals, 3 mL of 80% methanol was added to 0.15 g of green-chop powder, and the samples were shaken on a flatbed orbital shaker at 350 rpm for 1 h at 0 °C. After centrifugation (at 4000 *g* for 5 min), the supernatant was filtered through 0.45-µm syringe filter prior to LC-MS analysis of possible allelochemicals.

LC-MS analysis

Chromatographic separation for identification and quantification of allelopathic compounds was achieved using a Hypersil Gold column (150×2.1 mm, 1.9 µm, Thermo Scientific) on a Vanquish UPLC system (Thermo Scientific). The column compartment was maintained at 50 °C and the auto-sampler at 8 °C. The mobile phase was composed of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The flow rate was 0.25 mL/min with a gradient elution of 5 to 100% B over 20 min. The injection volume was 5 µL.

The detection of analytes was achieved using a Q Exactive Plus Orbitrap mass spectrometer (Thermo Scientific) operated in the electrospray ionization positive mode. The resolution was set to 70,000 for MS (parent ion) scan mode (100-750 *m/z*) and putative allelopathic compounds (maackian, pisatin and anhydropisatin) were extracted from the full-scan chromatograms using a mass tolerance window of 5 ppm; the relative abundance of these compounds in different samples was estimated by the extracted peak area. To confirm the identity of these putative compounds, data-dependent MS^2 spectra were acquired for selected parent ions (protonated molecular ions) at a resolution of 17,500 with a normalized collision energy of 50. Product ions were assigned manually based on the structures of the compounds and the accurate mass of the plausible fragments.

Statistical analysis

A two-way ANOVA with a blocking variable was conducted to assess the effect of different amendments and soil types on plant biomass, tiller number, and the concentrations of shoot N, P and K in Experiments 1 and 2. A one-way analysis of variance was used for Experiment 3 to compare the effects of different green-chop treatments on the wheat growth response. Significant (P=0.05) differences between means were identified using Turkey's test. The analyses were undertaken using Genstat (11th version).

Results

Experiment 1

Dry weights of the shoots and roots showed varying responses to the deep placement of the different amendments in three soils (Table 3). For example, the green-chop amendment did not affect the shoot and root dry weight in Chromosol and Sodosol but it doubled shoot biomass and tiller number that of the control in Vertosol. In contrast, the poultry litter and straw+NPKS amendments significantly increased shoot and root biomass and tiller numbers in all three soils. The straw amendment did not affect the wheat growth in any of the soils. This dependency of the growth response to green chop soil types, was responsible for the highly significant (P <0.001) amendment × soil interaction (Table 3).

Interactions between the amendments and soil types also occurred for the shoot N, P and K concentrations (Table 3). Poultry litter resulted in the highest shoot P concentrations with the Chromosol and Sodosol soils, but not in the Vertosol, where the straw+NPKS resulted in the highest P concentrations. The green-chop amendment resulted in the highest N and K shoot concentrations in all soils.

Experiment 2

A similar pattern of wheat growth responses to the deep placement of the green-chop amendment in three subsoils occurred in the second experiment. There was a trend for the shoot biomass of wheat plants to decrease, although not significantly so, when green chop was incorporated in the subsoils of the Chromosol and Sodosol without preincubation (Table 4). In contrast, shoot biomass increased by 60% for wheat plants grown in the Vertosol amended with green chop. Green chop with inorganic nutrients resulted in similar shoot responses to the green chop alone, in both Chromosol and Sodosol. Similar results were detected in tiller number.

Adding green chop to the Chromosol or Sodosol did not increase shoot N, P or K concentrations above the control, whereas adding green chop to the Vertosol increased the concentrations of all three elements above that of the control (Table 4). Addition of NPKS to the green chop generally increased the shoot N, P and K concentrations, although not all increases were significant.

Daily water use by wheat plants further reflected the impaired functioning of wheat plants growing in Chromosol and Sodosol subsoils amended with green chop (Fig. **1a, b**). These plants transpired considerably less water 25 days after sowing, than the plants from the control and NPKS treatments. In contrast, the wheat plants growing in the green-chop-amended Vertosol subsoils progressively increased their daily transpiration water loss up to around 200 mL column^{-1} from days 36 to 40, which exceeded the water use in the NPKS treatment (Fig. **1c**).

The initial transpirational loss of water after 20 days, from wheat plants in the green-chop-amended Vertosol soil in Experiment 1 with 4 weeks of pre-incubation was considerably larger (65-85%) than the initial transpiration water loss in Experiment 2 with no pre-incubation (Fig. **1c**). Cumulative transpirational water loss from the control was similar between two experiments. The rapid increase in water loss after 30 days, from the green-chop-amended Vertosol in Experiment 2, indicates that the constraints to wheat growth, associated with the absence of a pre-incubation, gradually diminished over time.

The green-chop-induced growth suppression in the Chromosol and Sodosol soils in Experiment 1 were associated with the almost complete cessation of root growth in the amendment layers (Fig. **2a, b**). There were practically no wheat roots in and below the green-chop bands in the Chromosol and Sodosol. However, adding green chop to the Vertosol subsoil resulted in 145% increase in root length density at the depth of 14-20 cm (Fig. **2c**). The root length density in the amendment layer was similar to that of NPKS treatments which generally had the greatest root length through the soil columns.

Soil microbial biomass C (MBC) in the Vertosol was nearly double that of in Chromosol and Sodosol (Fig. **3b**). The opposite occurred in the extractable soil organic C (EOC), which was 2 to 4-fold higher in Chromosol and Sodosol than in Vertosol. Both MBC and EOC showed an apparent increase in response to green chop and Green chop+NPKS, but little change to the NPKS treatment (Fig. **3a**).

Potential allelopathic compounds were identified from the extracts of the decaying green chop collected after 42 days. This was achieved by comparing the MS experimental mass/charge ratio (m/z ratio) with their calculated values (Fig. 4). The compounds with peaks occurring at m/z 285.0761, m/z 297.0760, m/z 315.0867 were tentatively identified as maackiain, anhydropisatin and pisatin, respectively. The identity of all these chemicals were confirmed by a MS/MS experiment with a fragmentation analysis. The data for this analysis are presented in Figure S1.

Except for pisatin, the relative concentrations of maackian and anhydropisatin were approximately 2 times higher in the green chop recovered from Chromosol and Sodosol than from the Vertosol (Fig. 5a). Pisatin, although present at relatively higher concentrations in the Vertosol soil, was 7-8 times less than

maackian in both Chromosol and Sodosol. Maackian and Pisatin were present in the original field pea shoot material at concentration 3-11 times higher than that recovered from all three soils (Fig. 5b). Noticeably, the concentration of all allelochemicals decreased by 70-80%, when green chop had been pre-incubated for 10 weeks.

Experiment 3

Adding activated carbon to the green chop placed in the Sodosol completely alleviated the growth suppression of the wheat plants. Shoot growth increased more than 2-fold, with root growth increasing 3 fold and the daily water use by 4 fold, compared with the green-chop-only treatment (Table 5). The 10-week pre-incubation of green chop in the Sodosol also increased shoot and root dry weights by 52% and 90%, respectively. Adding 3-cm sand or liming the soil layer above and below green-chop amendments, did not result in any increase in the final shoot and root biomass at day 42 (Table 5). However, sand addition did increase plant daily water use and the N concentration, as indicated by the SPAD reading, at Day 25.

Root proliferation in the green-chop-amended layer and in the surrounding subsoil increased markedly with the addition of activated carbon (Fig. 6). Total root length density in and below the amendment layer, in the presence of activated carbon, was 2.4-3.5 fold higher than that in the control. The 10-week pre-incubation increased root length density by 90% in the subsoil above the control. The lime treatments showed little effect on the root growth in and below the amendment layer, when compared with the green-chop-only treatment.

Discussion

Significant differences in wheat growth response, to the deep placement of the green-chop amendment, were detected among the three soils assessed. In particular, root growth into the green-chop bands and surrounding subsoil was completely suppressed in the Chromosol and Sodosol subsoils; in contrast deep root proliferation was greatly enhanced in the Vertosol (Fig. 2). This inhibitory effect of green chop on wheat growth in the Chromosol and Sodosol soils is attributed to phytotoxic chemicals released from the decomposing green chop. Activated carbon has been well recognized for its capacity to adsorb and neutralize phytotoxic organic molecules (Hille and Den Ouden 2005; Bonanomi et al. 2011). Adding activated carbon to the green-chop amendment greatly alleviated the growth suppression of wheat plants (Table 5, Fig. 6), which might provide additional evidence for the allelopathy hypothesis. It appears that the toxic effects of decaying green chop were largely affected by the physicochemical environment in the subsoil. Understanding the impact of the green chop on wheat growth needs to consider how subsoil conditions affected the microbial breakdown of all allelochemicals.

In this study, the inhibitory effect of green chop on wheat growth in the Sodosol and Chromosol could not be attributed to low nutrient availability for a number of reasons. Firstly, the relatively low C:N ratio of 14.7 in the green chop should favor net microbial N mobilization (Hodge et al. 2000; Bonanomi et al. 2010). Secondly, the P concentration of 3.1 mg g^{-1} in the green chop was above the level of 2 to 3 mg g^{-1} and should not led to net P immobilization (Yadvinder-Singh et al. 1992). Thirdly, although nutrient release rates from the green chop were expected to be slower in the Sodosol and Chromosol subsoils with lower soil pH, lower air-filled porosity and microbial biomass, the addition of inorganic nutrients with the green chop did not remove the growth constraints on wheat plants. Finally, root growth retardation in the subsoil in the presence of green chop was not detected in the control with no amendments, further suggesting that the inhibitory factor was not related to nutrient deficiency.

The identification of allelochemicals in the decomposing green-chop material provides strong support for phytotoxicity as the cause for the inhibition of wheat growth in the Chromosol and Sodosol. All of the identified allelochemicals were present in the original green-chop material in relatively high concentrations, indicating that the green chop was the source of these allelochemicals. Allelopathic effect of wheat straw, though reported by other studies (Zuo et al. 2008; Wu et al. 2007), was not detected in the first experiment

with one-month pre-incubation. This is consistent with findings that N_2-fixing species, such as legumes, were more toxic than grass species such as wheat (Miller, 1996; Rice et al. 2004; Bonanomi et al. 2006). Although hundreds of phytotoxic organic molecules have been reported in plant litters (Armstrong and Armstrong 2001; Kraus et al. 2003; Reigosa et al. 2006), only a limited number of allelochemicals have been extracted and identified in the shoots of field pea (Carlson and Dolphin 1981; Kato-Noguchi, 2003; Arman, 2011). In line with early studies, pisatin and maackiain were two major phytoalexins detected in the field-pea shoots in this study, in addition to their precursors such as pseudobaptigenin (data not shown) (Harborne, 2013). Particularly, pisatin is a well-known antifungal molecule produced naturally in tissues of field pea (Cruickshank and Perrin 1960; Carlson and Dolphin 1981; Kato-Noguchi, 2003). The complete cessation of root growth into the green-chop bands was possibly attributed to the disintegration of the root cap or altered membrane function in the root cells in the presence of these allelochemicals (Curlango-Rivera et al. 2010; Fageria and Baligar 2003; Rugare et al. 2018). As no attempt was made to identify new allelochemicals, the possibility that other allelochemicals were present in the field-pea residue cannot be ruled out.

The higher concentrations of total allelochemicals in the Chromosol and Sodosol than the Vertosol were consistent with the inhibitory effect of green chop on wheat growth in these two soils. The only exception was pisatin which was present at a relatively higher concentration in the Vertosol than in the Chromosol or Sodosol subsoils. This can be explained by the fact that pisatin is relative stable at neutral and alkaline pH, but tends to form anhydropisatin under acidic conditions with the loss of a water molecule (Perrin and Bottomley 1962). Consistently, higher concentrations of anhydropisatin were measured in green chop recovered from the Chromosol and Sodosol subsoil, compared to Vertosol and the original green-chop material. It has been noted by Bonanomi et al. (2011) that the observed phytotoxicity from decaying plant shoots results from both the total concentration of all allelochemicals and their synergistic effects. The total concentration of these allelochemicals was more than two times higher in the Chromosol and Sodosol, than in the Vertosol, accounting for their pronounced suppressive effect on wheat root growth.

Differential expressions of phytotoxicity in three soils might be attributed to difference in soil properties such as pH, texture and/or soil aeration. All allelochemicals, once released into the soil, were subjected to various detoxification processes such as adsorption by soil organic matter and/or clay minerals, microbial decomposition or auto-polymerization (Bollag 1992; Blum et al. 1999; An et al. 2001; Ehlers, 2011). In this study, detoxification of allelochemicals in the Vertosol via soil absorption should be less significant, considering that green chop was applied in a concentrated band, not mixed with soil. Actually, noticeable root proliferation occurred within the green-chop band in the Vertosol, in contrast to the Sodosol and Chromosol where plant roots actually failed to penetrate the amendment band. On the other hand, increasing subsoil pH from 4.8 to 6.5 by liming did not alleviate the phytotoxicity of green chop in Sodosol (Table 5, Fig. 6), indicating that reduced phytotoxicity in the Vertosol might not be attributed to higher soil pH. However, the presence of a sand layer did temporarily reduce the inhibitory effect of green chop on wheat growth at the early stage (before 20 days). The physical properties of the Chromosol and Sodosol subsoils, when compacted to a high bulk density (1.4 g cm^{-3}) and watered frequently to field capacity, were likely to promote anaerobic conditions (MacEwan et al. 2010). In addition, the low air-filled porosity in both topsoil and subsoil (Table 1) would result in reduced air diffusion from the atmosphere to the amendment bands. Likely, it is anaerobic conditions in the Chromosol and Sodosol subsoils that resulted in decreased microbial growth or activity, and hence accumulation of allelochemicals to a phytotoxic level. This is supported by numerous reports that anerobic conditions produced stronger and more persistent phytotoxic level (Patrick, 1971; Bonanomi et al. 2006). The air retained in the 6-cm sand layer would have been gradually depleted during the decomposition of the green chop, which would explain the lack of benefit from the sand layer after 20 days.

Decreased phytotoxicity, with extended pre-incubation (Table 5, Fig 2), can be explained by the continuing microbial breakdown of the allelochemicals. The allelochemicals, sourced from readily-

decomposable C fractions of plant litter (Bonanomi et al. 2011), would be subject to rapidly detoxification by soil microbes (Schmidt and Lipson 2004; Kaur et al. 2009). The phototoxicity of plant materials has been noted to diminish rapidly after several days (Ohno et al. 2000; Xuan et al. 2005; Bonanomi et al. 2006) or several weeks (Bonanomi et al. 2011). Previous studies found that 3-4 weeks are necessary to reduce the phytotoxicity of plant materials to non-toxic concentrations when they were placed on the soil surface (Conn and Dighton 2000; Bonanomi et al. 2011). In this study, the positive growth response to deep placement of the green chop in the Vertosol was delayed by 30 days when there was no pre-incubation (Fig. 2c). Thus, it appears that a 4-week time period also applied when the green chop was incorporated in the well-structured Vertosol subsoil. In the Chromosol and Sodosol soils with poorly-structured subsoil and associated anaerobic conditions, the microbial decomposition of allelochemicals was likely to be constrained and slowed down. This is supported by the higher levels of allelochemicals in the decaying green-chop residues, higher concentration of the extractable organic C and the lower microbial biomass in soils adjacent to the amendments (Fig. 4). Under these conditions, a longer time period is required to completely remove the detrimental effects of phytotoxic chemicals on plant growth. An earlier preliminary study revealed that wheat plants were able to recover from the phytotoxicity 90 days after the deep incorporation of green chop in the Sodosol (data not shown). Further studies are needed to measure the time period required for the disappearance of the phytotoxic effects, and to relate this time period to the physical-chemical properties of the subsoil.

Conclusions

The inhibitory effect of the green chop on wheat growth in the Sodosol and Chromosol subsoils might be attributed to the phytotoxicity from allelochemicals that persisted under anaerobic conditions, rather than to a reduced nutrient supply or a low soil pH. This study suggests that phytotoxic effects of potential sources of green chop should be considered in addition to their nutritional composition. Caution is required in the field when green-chop material is incorporated into poorly-structured soils. Also, there is a risk of rainfall events, leading to possible waterlogging and anaerobic conditions in the subsoil. Land managers should plan for an extended time period, between incorporation of a field-pea-type residue and sowing, to reduce the risk of growth suppression in the following crop.

Acknowledgements

This research was supported under Grains Research and Development Corporation Projects funding scheme (project DAV00149).

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Table 1. Basic properties of topsoil (0-10 cm) and subsoil (10-45 cm) for Chromosol, Sodosol and Vertosol soils used in the study.

Soil	layer	pH (0.01 M $CaCl_2$)	EC (dS m^{-1})	Organic C (mg g^{-1})	NO_3^- +NH_4^+ (µg N g^{-1})	Olsen P (µg g^{-1})	Clay (%)	Air porosity (%)	Exchangeable Na (% of cations)
Chromosol	Top	4.0	0.14	49.1	75.2	16.7	18.7	18.1	0.7
	Sub	4.7	0.05	14.2	17.7	4.7	21.3	13.0	1.5
Sodosol	Top	4.3	0.29	38.3	20.2	29.9	21.0	13.2	9.2
	Sub	4.8	0.06	8.6	4.3	5.8	25.6	12.0	11.0
Vertosol	Top	7.4	0.17	9.2	8.8	26.9	43.7	26.1	0.9
	Sub	7.4	0.17	8.9	6.6	19.4	48.1	25.8	1.1

EC, electrical conductivity measured in 1:5 soil:water
Air porosity, air-filled porosity at field capacity

Table 2. Basic chemical properties for field pea shoot (green chop), wheat straw, poultry litter and activated charcoal used as soil amendments.

	Total N (mg g^{-1})	Total P (mg g^{-1})	Total K (mg g^{-1})	C:N ratio
Green chop (GC)	26.5	3.0	20.9	14.7
Wheat straw	2.5	0.4	7.2	165.2
Poultry litter	45.0	17.0	27.0	7.6
Activated charcoal (AC)	1.4	0.3	2.7	410.1

Table 3. Dry weights of shoot and root, tiller number and the concentrations of N, P and K in the shoot of wheat grown for 42 days in Chromosol, Sodosol and Vertosol soils amended with various amendments. The soil columns were incubated for 30 days before sowing (Experiment 1).

Soil types	Amendments	Biomass (g column^{-1})		Tiller No	Nutrient concentration (mg g^{-1})		
		Shoot	Root	(No column^{-1})	N	P	K
Chromosol	Control	3.43 a	0.97 a	3.2 a	34.3 g	2.65 b	32.4 efg
	Green chop	3.04 a	0.66 a	2.7 a	32.7 fg	2.85 b	34.2 fg
	Wheat straw	3.40 a	0.91 a	3.1 a	32.1 fg	2.88 bc	36.1 g
	Wheat straw + NPKS	12.01 ef	3.17 defg	7.3 f	19.9 e	3.03 bcd	33.6 fg
	Poultry litter	13.35 f	3.61 fgh	7.9 f	17.8 de	3.26 cd	30.6 def
Sodosol	Control	5.62 bc	2.21 b	3.8 b	18.4 de	1.90 a	21.6 a
	Green chop	6.39 bc	2.32 b	4.2 b	30.1 f	1.87 a	35.5 g
	Wheat straw	5.58 b	2.03 b	3.1 a	16.3 cd	2.01 a	28.6 cd
	Wheat straw + NPKS	10.96 de	3.03 cdef	5.0 cd	16.7 cd	2.85 b	29.1 cde
	Poultry litter	11.90 ef	3.67 gh	6.2 e	16.3 cd	3.36 d	27.0 bc
Vertosol	Control	7.15 c	2.57 bcd	4.3 b	14.2 abc	2.71 b	26.4 bc
	Green chop	16.17 g	4.07 h	9.9 g	19.6 e	3.40 d	35.9 g
	Wheat straw	7.01 bc	2.46 bcd	4.4 bc	12.1 a	2.70 b	23.4 ab
	Wheat straw + NPKS	9.56 d	2.99 cde	5.3 d	13.3 ab	4.06 e	30.8 def
	Poultry litter	11.30 e	3.44 efgh	7.7 f	15.7 bcd	2.72 b	22.5 a
Two-way ANOVA							
Soil types		***	***	***	***	***	**
Treatments		***	***	***	***	***	***
Soil types × Treatments		***	***	***	***	***	**

**, $P<0.01$; ***, $P<0.001$. For each column, different letters indicate significant differences between means (two-way ANOVA, Tukey test, $P<0.05$).

Table 4. Dry weights of shoot and root, tiller number and the concentrations of N, P and K in the shoot of wheat grown for 42 days in Chromosol, Sodosol and Vertosol soils amended with various amendments (Experiment 2).

Soil types	Treatments	Biomass (g column^{-1})		Tiller No	Nutrient concentration (mg g^{-1})		
		Shoot	Root	(No column^{-1})	N	P	K
Chromosol	Control	4.46 a	1.64 a	5.0 abc	34.1 def	2.75 de	24.9 de
	Green chop	3.60 a	1.68 a	4.3 abc	38.9 fg	2.22 bc	26.4 e
	Green chop + NPKS	3.61 a	1.54 a	4.0 a	40.9 g	2.50 cd	28.6 f
	NPKS	8.70 bc	3.16 de	7.0 d	37.4 efg	3.19 fg	28.6 f
Sodosol	Control	4.27 a	2.39 bc	5.2 bc	28.9 c	2.16 abc	23.7 d
	Green chop	3.60 a	2.33 bc	4.3 abc	32.9 cde	1.84 a	22.9 cd
	Green chop + NPKS	3.07 a	2.07 ab	4.2 ab	38.1 fg	1.98 ab	24.8 de
	NPKS	9.09 c	3.72 e	7.3 de	32.6 cd	3.43 gh	24.3 d
Vertosol	Control	7.15 b	2.64 cd	5.3 c	15.6 a	2.71 de	12.7 a
	Green chop	11.34 d	3.09 d	8.0 def	22.0 b	3.70 hi	21.4 bc
	Green chop + NPKS	11.46 d	2.92 d	8.3 ef	32.0 cd	3.80 i	24.1 d
	NPKS	14.61 e	4.90 f	8.7 f	18.3 ab	3.06 ef	19.9 b
Two-way ANOVA							
Soil types		***	***	***	***	***	***
Treatments		***	***	***	***	***	***
Soil types × Treatments		***	***	***	***	***	***

**, $P<0.01$; ***, $P<0.001$. For each column, different letters indicate significant differences between means (two-way ANOVA, Tukey test, $P<0.05$).

Table 5. Dry weights of shoot and root, the concentrations of N in the shoot, daily water use, and leaf SPAD reading of wheat grown for 42 days in Sodosol with various treatments (Experiment 3).

Treatments	Biomass (g column^{-1})		Shoot N concentration (g mg^{-1})	Daily water use (ml column^{-1})		Spad reading	
	Shoot	Root		Day 25	Day 45	Day 25	Day 45
GC (Green chop only)	5.4 a	2.1 a	28.8 a	60 a	53 a	44.1 a	48.3 a
GC + lime	5.6 a	2.1 a	29.8 a	63 a	52 a	45.2 a	49.3 ab
GC + sand	5.9 a	3.0 b	34.6 b	76 b	65 a	51.1 b	50.7 a
GC + 10-week incubation	8.2 b	4.0 c	36.6 b	78 b	175 b	51.0 b	50.9 ab
GC + Activated carbon	12.4 c	6.1 d	28.1 a	84 b	245 c	52.5 b	53.0 b

GC, Green chop.

Means followed by different letters differ significantly ($P<0.05$), as determined by one-way analysis of variance using Tukey's multiple range test.

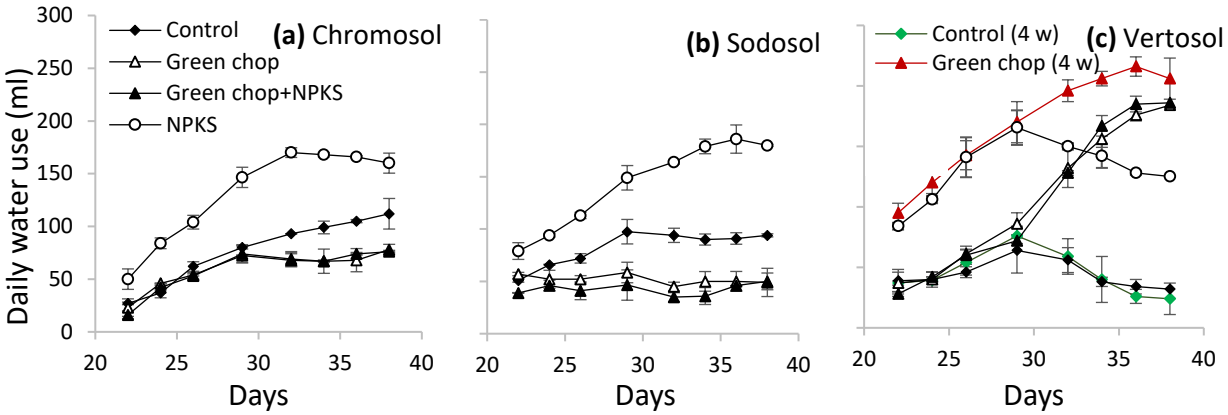

Fig. 1. Daily water use of wheat plant grown in Chromosol (a), Sodosol (b) and Vertosol (c)without (Control) and with incorporation of green chop, green chop+NPKS and NPKS into the subsoil (at 17 cm) with no pre-incubation (Black line) and with 4 weeks of preincubation (4w, red and green line) in green-chop amended Vertosol (Experiment 1). Error bars represent ± the standard error of three means.

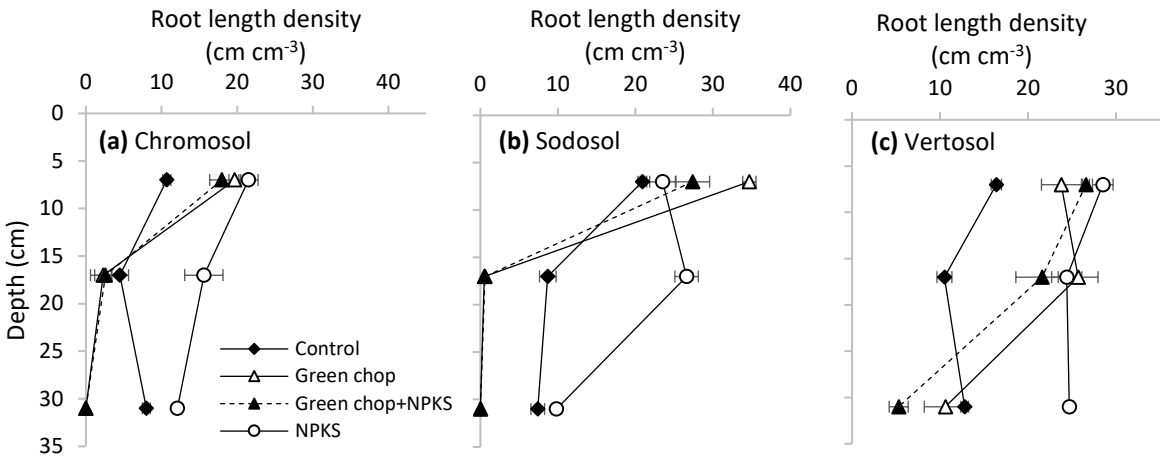

Fig. 2. Root length density of wheat plant grown for 42 days in Chromosol (a), Sodosol (b) and Vertosol (c) without and with incorporation of green chop, green chop+NPKS and NPKS into the subsoil (at 17 cm), (Experiment 2). Error bars represent ± the standard error of three means.

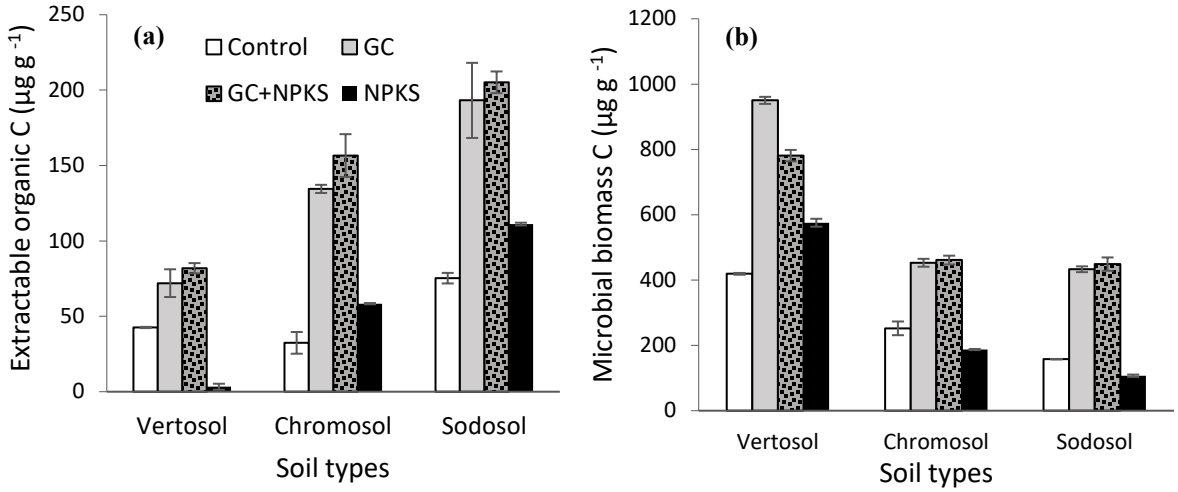

Fig. 3. Extractable organic C (EOC) and microbial biomass C in soil layers proximate to the amendments (green chop, green chop+NPKS and NPKS) in the subsoil layers in the Chromosol, Sodosol and Vertosol soils (Experiment 2). Error bars represent ± the standard error of three means.

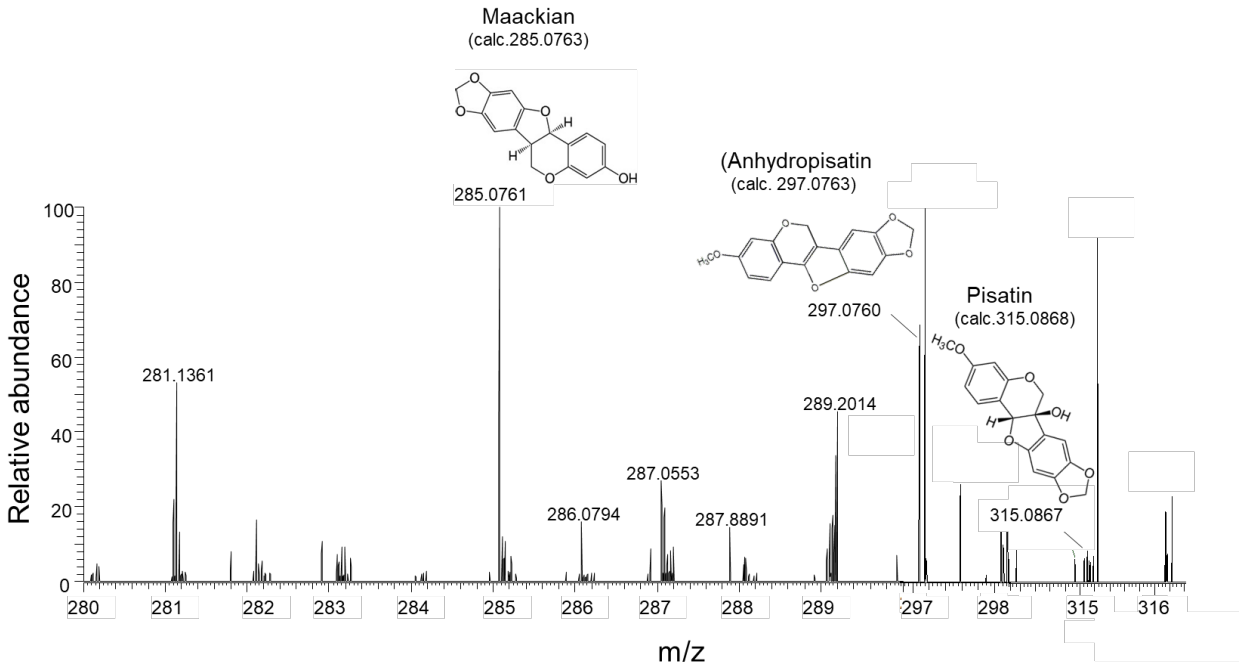

Fig 4. The identified allelopathic chemicals: macckian, anhydropisatin, pisatin from decaying green chop. The peaks are labelled by their chemical structure and mass/charge (m/z) ratio.

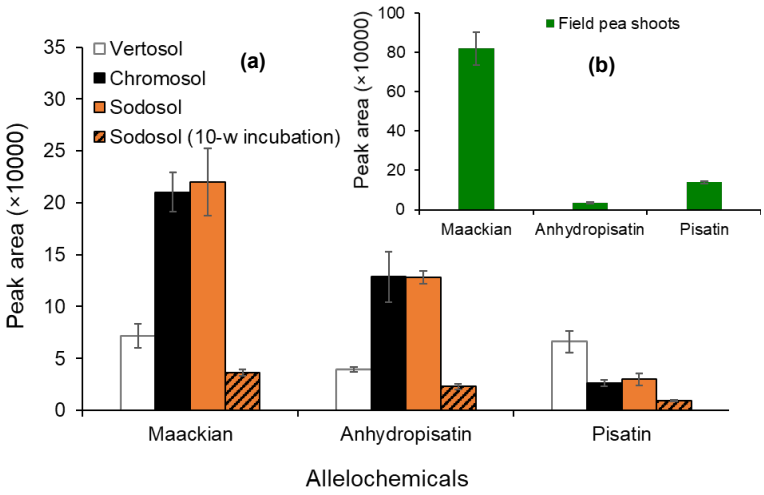

Fig. 5. The relative concentrations of three phytotoxic chemicals (maackian, anhydropisatin, pisatin) in field pea shoots (b), and the decaying green chop recovered from Chromosol, Sodosol and Vertosol subsoils after 42 days of wheat growth (Experiment 2) (a).

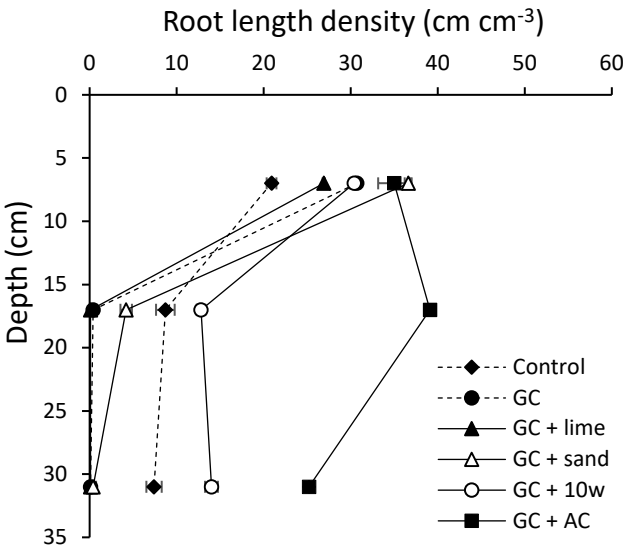

Fig. 6. Change in root length density of wheat plant in response to green chop (GC) added into the subsoil of a Sodosol without (control) and with different treatments (+lime, + sand, +10w pre-incubation, +activated carbon (AC) (Experiment 3).

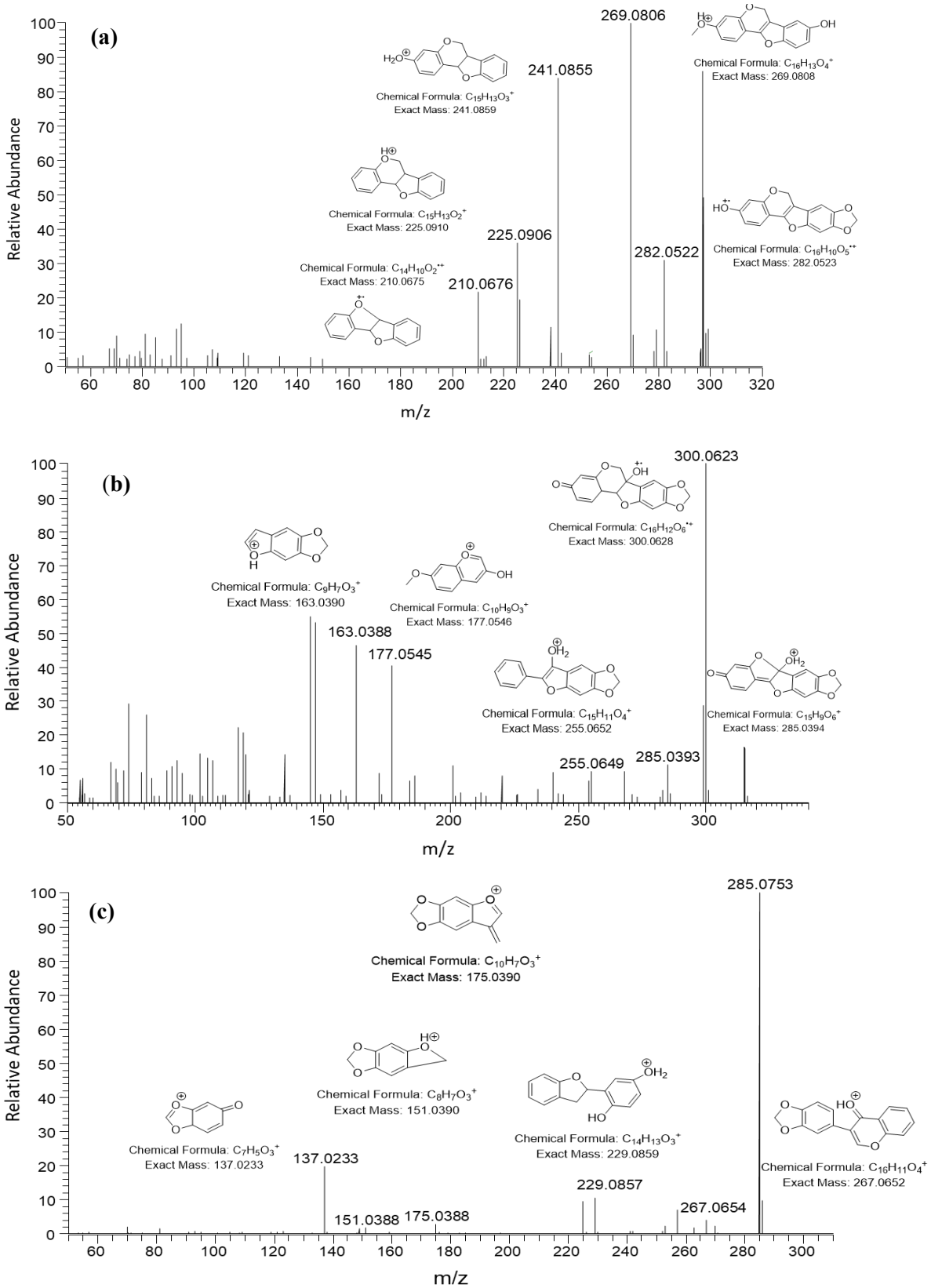

Fig. S1. Major product ions and tentative assignment of pisatin (a), anhydropisatin (b) and maackiain (c) identified in field pea residue.